CROSS-SECTIONAL STRUCTURE AND VALIDATION OF THE TIMING OF ANNULUS FORMATION IN OTOLITHS OF THE ANTARCTIC FISH *NOTOTHENIA CORIICEPS* RICHARDSON (NOTOTHENIIDAE)

by

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ABSTRACT. - To validate the timing of annuli in otoliths of immature *Notothenia coriiceps* Richardson, a time-series of samples were taken over a complete year. Light and scanning electron microscopy (SEM) techniques were used to examine the structure of sectioned otoliths. Six growth regions were identified in the otolith sections and micro-increments were also evident. The timing of growth and annual nature of annuli revealed by SEM were demonstrated. Annuli revealed by SEM and light microscopy techniques were shown to correspond, supporting the hypothesis that annuli visible by using light microscopy represent one year. Using SEM the potential errors due to light illumination artefacts and the pseudo-hyaline features could be avoided.

RÉSUMÉ. - Pour valider le rythme de formation des annuli des otolithes d'individus immatures de *Notothenia coriiceps* Richardson, une série d'échantillons, prélevés régulièrement dans le temps pendant un an, a été utilisée. Les techniques de microscopie photonique et de microscopie électronique à balayage (MEB) ont été utilisées pour examiner des coupes d'otolithes et en comparer les structures. Six régions structurales ont été identifiées sur les coupes et des micro-accroissements ont aussi été mis en évidence. Le rythme de la croissance et la nature annuelle du dépôt des annuli révélés par le MEB ont été démontrés. Les structures révélées par le MEB et celles qui ont été observées en microscopie photonique sont superposables, ce qui supporte l'hypothèse qu'un annulus visible en microscopie photonique représente une année de vie. En utilisant le MEB, les erreurs potentielles dues à l'éclairage et les images pseudo-hyalines pourraient être supprimées.

Key-words. - Nototheniidae, *Notothenia coriiceps*, PS, Antarctic ocean, Otoliths, Validation, Scanning Electron Microscope, Age estimation.

Large-scale fisheries in the South Atlantic sector of the Antarctic first started at South Georgia in 1969, extending to the South Orkney Islands in the austral summer of 1977/78 and thence to the South Shetland Islands and northern Antarctic Peninsula in 1978/79 (Hureau and Slosarczyk, 1990; Kock and Koster, 1990). The initial target species at South Georgia was the nototheniid, *Notothenia rossii marmorata*, closely related to *Notothenia coriiceps* (formerly known as *Notothenia neglecta*). With the collapse of this fishery in 1971, emphasis shifted to the channichthyid *Champsocephalus gunnari* and later to the nototheniid, *Patagonotothen brevicauda guntheri*; by-catch species have included *Gobionotothen gibberifrons* and *Lepidonotothen squamifrons*. In the smaller fishery at the South Orkney Islands, activity has been directed at *C. gunnari*, with *G. gibberifrons* and to a lesser extent *N. rossii* as by-catches. Unidentified species have also formed a large part of the catch and Kock *et al.* (1985) concluded that an unknown

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proportion of catches from the Antarctic Peninsula may consist of *N. coriiceps*, misidentified due to its similarity to *N. rossii*.

Management of Antarctic fish stocks is based largely upon age-dependent models that require reliable age estimation techniques: small inaccuracies have been demonstrated to have large effects on demographic predictions used in decision-making (Coggan *et al.*, 1990). Structures showing growth increments have been found in the otoliths of Antarctic fish (North *et al.*, 1980; Townsend, 1980; Everson, 1981; Daniels, 1983; Radtke *et al.*, 1989; Coggan *et al.*, 1990) and have been used for age estimation (e.g., Burchett, 1983a, 1983b; Daniels, 1983; Barrera-Oro and Casaux 1992), although few studies have attempted to validate these structures (North, 1988). The international oceanographic study BIOMASS (Biological Investigations of Marine Antarctic Systems and Stocks) and the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) have emphasized the importance of reliable techniques for age determination and CCAMLR has organized a series of workshops held by the Working Group for Fish Stock Assessment (Anon., 1989). Kock (1989) reported the results of a study to test the consistency of age estimates by inviting different scientists to read a set of otoliths and scales. He showed that there was considerable variation in the ages determined by different readers and no better agreement among 'experienced' investigators than less experienced ones: it was concluded that more validation studies were needed. White (1991) concluded that the analysis of otoliths was the most reliable and accurate method for age estimation of Antarctic fish, and reiterated the need for validation studies.

Radtke and Hourigan (1990) used tetracycline and acetazolamide marking techniques to demonstrate that otolith micro-increments were deposited daily in *Lepidonotothen nudifrons*. Larger patterns were also revealed using the Scanning Electron Microscopy (SEM) but they concluded that these were not true annuli because they comprised too few micro-increments to be directly related to an annual period.

The technique of sampling at regular intervals throughout the annual cycle has not been widely applied to validate age structures in Antarctic fish because of the problems of making collections during the winter. Burchett (1983b) indicated the annual nature of annuli revealed by light microscopy in *N. rossii* at South Georgia. North (1988) using light microscopy, to examine the timing of annuli formation in otoliths and scales across an assemblage of fish species from South Georgia and the South Orkney Islands found evidence for the hypothesis that an annulus represents a year in the life history of Antarctic fish. North (1988) further demonstrated the presence of a pseudo-hyaline edge which introduced an error of about one month in reading the stage of growth of otoliths.

The present study was undertaken to validate the timing of annulus growth in *N. coriiceps*; a species that is readily accessible during the whole year from the British Antarctic Survey research station at Signy Island, South Orkney Islands, Antarctica (60° 42'S, 45° 36'W). The biology and population dynamics of *N. coriiceps* at the same locality has been well described by Everson (1970). These factors, together with its close relationships with several exploited species subject to active commercial-scale fishing (e.g., *Notothenia rossii*, *Gobionotothen gibberifrons*, *Lepidonotothen squamifrons*), and its possible role as a by-catch (Kock *et al.*, 1985), made *N. coriiceps* an appropriate species for a study to validate seasonal growth structures by a 'time-series' investigation.

MATERIALS AND METHODS

Immature *N. coriiceps* of mean standard length 23.6 cm (sd ± 3.9 cm) were sampled at Signy Island, from two inshore sites at 9 m depth in Factory Cove and further off-shore in 18 m in Borge Bay (Fig. 1). Regular samples of fish were collected at approximately monthly intervals using a 30 m long trammel net, set overnight and recovered the following morning or as weather conditions allowed. Ten to 20 juvenile *N. coriiceps* were selected from the catches. Nets were set using a boat during summer and

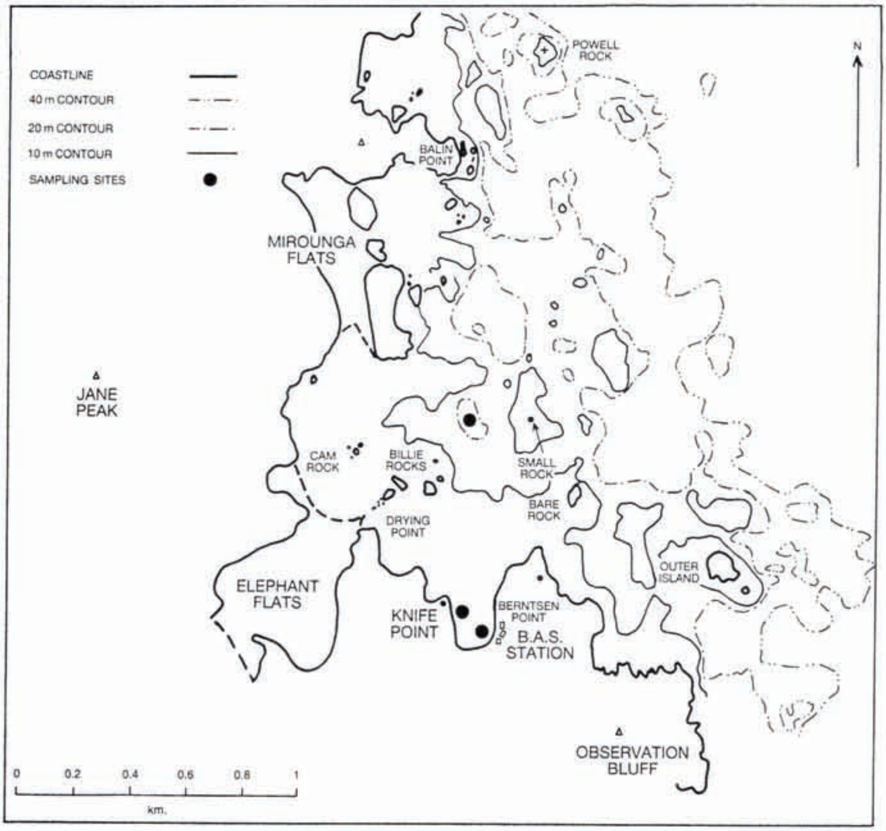


Fig. 1. - Borge Bay, Signy Island, showing locality of research station, bathymetry and sampling sites.

in winter by swimming along a line between two holes in the sea-ice using SCUBA techniques. One sample, taken on 2 June 1988 was caught by hook and line in 6 m depth by the research station jetty in Factory Cove. Logistic commitments resulted in the December collection being made late in the month and so the January sample was used. No samples were collected during March and April owing to extreme inclement weather.

The fish were sacrificed and biometric data taken. Otoliths were extracted, washed in a dilute solution of detergent, rinsed and left to dry before being stored in labelled vials. Otoliths from *N. coriiceps* are small, usually < 4 mm long.

Otoliths were prepared for light microscopy examination by embedding them in coloured polyester resin and then cutting 0.5 mm thick thin-sections in the manner developed by Bedford (1983). To assist with interpretation the otoliths were oriented as uniformly as possible. Where possible, the right otolith was processed. The longitudinal axis of each otolith was placed parallel to the long axis of the mould with the sulcus side flat to the resin. Structures on sections from each specimen were examined using both reflected and transmitted light to determine the stage of growth reached by the outer annulus.

The terminology for otolith structures found using light microscopy follows the nomenclature of Everson (1981) for Antarctic fish. The internal structure of an otolith consists of a series of (usually concentric) annuli, proceeding away from a central

nucleus. Annuli in turn are divided into a hyaline zone of material which permits the passage of light and an opaque zone which obstructs light when viewed in transmitted light. When using Scanning Electron Microscopy (SEM) techniques a complete annulus comprised a broad lightly-etched zone and a narrow heavily-etched zone. Fine-scale concentric structures such as micro-increments follow the terminology of Pannella (1971).

Sections examined using light microscopy were classified according to the system adopted by North (1988) for hyaline zone, opaque zone and pseudo-hyaline edge; they were divided into two categories according to whether opaque or hyaline zones were present on the outer edge of the otolith and then sub-divided into arbitrary 'narrow' and 'well-developed' categories to indicate development of each zone. The division between the terms 'narrow' and 'well-developed' was on the basis of the structure being less or greater than 50% of the width of the same structure in the adjacent whole annulus.

To circumvent the problems raised by North (1988) using light microscopy, specimens were also prepared for viewing by SEM using the method of Ashford *et al.* (1993). The etching agents, hydrochloric acid and EDTA at a range of dilutions and durations were used in initial trials. Etching with EDTA was found to produce the best relief across the whole otolith section. An optimum result between effectively resolving the fine detail and over-etching the larger-scale structures was achieved by varying the concentration of the etching solution and duration of the treatment. After experiment, a routine was adopted where otoliths sectioned to reveal the nucleus were polished, etched in 4% EDTA for five minutes, then rinsed and immersed for at least five minutes in distilled water.

After etching, the blocks containing the sectioned otoliths were dried, mounted on SEM stubs using epoxy resin or double-sided adhesive tape, coated with gold using a BIORAD SC502 sputter-coating unit, and earthed to the stub using colloidal silver. The otolith sections were then viewed using a Leica Cambridge 360 SEM. For each otolith, the SEM image processing facility was used to produce stored images, using the line integrate function. Videoprints were taken of the whole otolith section at x 75 magnification and a previously selected edge region at x 200. The outer edge of the otolith was examined to determine the structures present without reference to the date of sacrifice to avoid subjective assessments. Videoprints were also taken of a sub-sample of otoliths under a light microscope before etching, to compare with structures observed using the SEM.

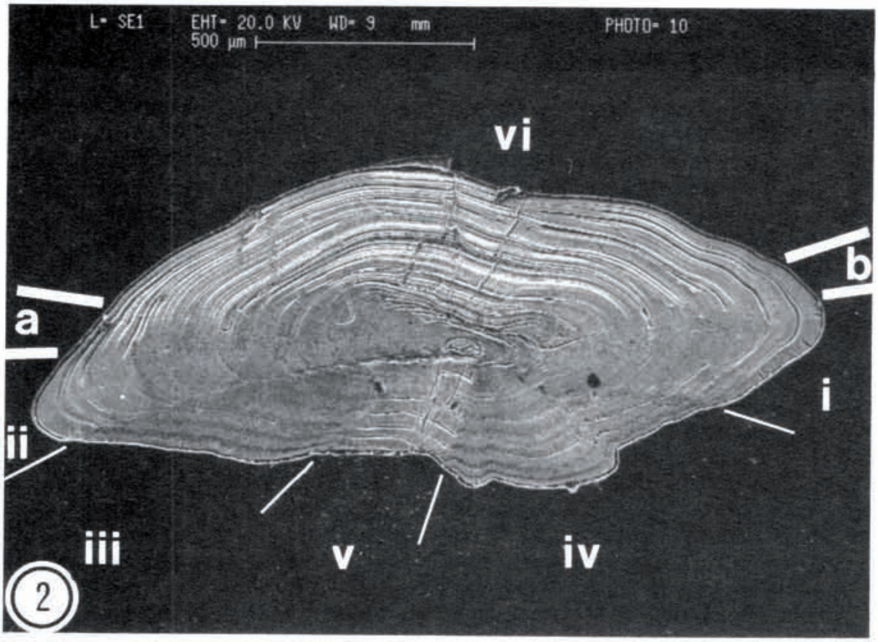
In specimens where the resin had infiltrated the otolith, the true edge was detected by measuring calcium levels using the energy dispersal x-ray function of the SEM.

RESULTS

Otolith structure in cross-section

Transverse cross-sections of *N. coriiceps* otoliths were found to have a highly differentiated structure when viewed using a SEM (Fig. 2). Typically, six regions could be distinguished, radiating from the centre of the otolith. Annuli were also revealed by SEM as a pair of adjacent bands, one heavily-etched and the other lightly-etched. The structure of these bands were modified characteristically within each region. Boundaries between regions were marked either clearly by a fusion line or by a gradual transformation of growth patterns. Within the first annulus was a distinct nuclear core with no sign of regional boundaries separated from the rest of the otolith by a distinct check.

In cross-section, two regions (Fig. 2 i, ii) extended respectively dorsally and ventrally from the central nuclear core with wide annuli divided into wide lightly-etched and narrower heavily-etched bands. On the interior side of the section another two regions (Fig. 2 iii, iv) extended from the nucleus on either side of a fifth region formed by the bridge of the sulcus: these were characterized by narrow annuli in which the



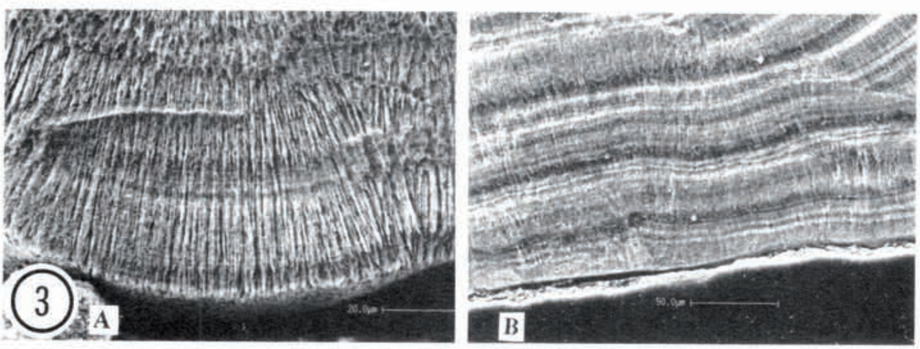


Fig. 2. - SEM photomicrograph of transverse section of otolith from *Notothenia coriiceps* (10^+ years) showing differentiated regions of growth and boundaries. Thin lines denote fusion zones between regions of growth on inner side of otolith. a and b mark boundaries between regions demarcated by a gradual transformation of growth patterns. Thick lines denote limits of these changes in pattern. Regions of growth are marked i-vi, for further detail see text.

Fig. 3. - SEM photomicrograph comparing growth stage in region iii of the outer annulus from *Notothenia coriiceps* otolith section. A shows otolith with well developed heavily-etched band at the otolith edge (end of stage 2); B shows otolith with a well developed lightly-etched band at the otolith edge (stage 4).

lightly-etched bands were frequently of a similar width as the heavily-etched bands. The sixth region was on the exterior side with narrow annuli divided into wide lightly-etched and narrow heavily-etched bands.

Micro-increments within the annuli revealed by SEM were evident, notably around the central nuclear core. The width of micro-increments varied from 0.4 µm to 1.9 µm.

The width of annuli revealed by both light and SEM microscopy decreased visibly from the centre to the outer edge of the otolith. For example, the width of the outer 3 complete annuli were measured along the exterior-lateral axis in a subsample of 5 specimens and these decreased from 120.2 µm (sd ± 25.7) to 85.5 µm (sd ± 18.7) to 73.7 µm (sd ± 16.4) from inner to outer respectively.

Validation of annual structures

Interpretation of sections viewed by light microscopy were equivocal. This was due to a number of factors that made several different interpretations possible: a) the small size of the otoliths; b) the narrowness of the annuli observed; c) aberrations at the otolith edge when using light microscopy; d) the frequent presence of a pseudo-hyaline edge; e) variations in opacity of the opaque zone.

Of 82 otoliths examined, 11 (13%) were unreadable; and of those read, 36 (51%) were considered doubtful in at least one repeat reading. Variations between readings were high [29 of 71 (39%) varying by one category or more] with dramatic variations between monthly samples: all repeat readings for otoliths taken on 13 July agreed, whereas those taken on 6th January, no repeat readings agreed where the otolith was readable.

The results using SEM were more readily interpreted. In addition, examination by SEM allowed any infusion of the outer otolith layer with stained polyester to be corrected for. Confirmation of the true edge was achieved with the SEM by checking calcium levels using energy-dispersive X-ray analysis.

Analysis of the outermost annulus in region iii revealed by SEM was based on four categories adopted from North (1988): videoprint images were divided according to whether lightly or heavily-etched bands were present on the outer edge of the otolith and then sub-divided into narrow or well-developed categories to indicate development of each zone (Fig. 3). The observation of these structures gave clear results indicating that the heavily-etched bands started appearing on the edge of the otoliths in September (winter), progressing to become well-developed by the beginning of February (summer). The lightly-etched band first appeared at the periphery from between early February and early May in 1988, and was present in most specimens from 5 June 1987 until 11 August 1987, and from 3 May 1988 until sampling ended on 25 June 1988. The progression of stages is shown in figure 4. The statistical distribution of stages using pooled data for analysis (Table I) was significantly different from uniform according to a chi-square test (9 degrees of freedom, $p < 0.001$). This consistent seasonal development demonstrated that the annuli visible using the SEM represented one year of growth.

Table I. - Frequency of otoliths within each growth stage from observations of outer annulus using pooled data.

Period	Stage	1	2	3	4
June - August 1987		1	0	10	19
September - November 1987		13	2	1	18
January - February 1988		5	12	1	0
May - June 1988		1	0	15	15

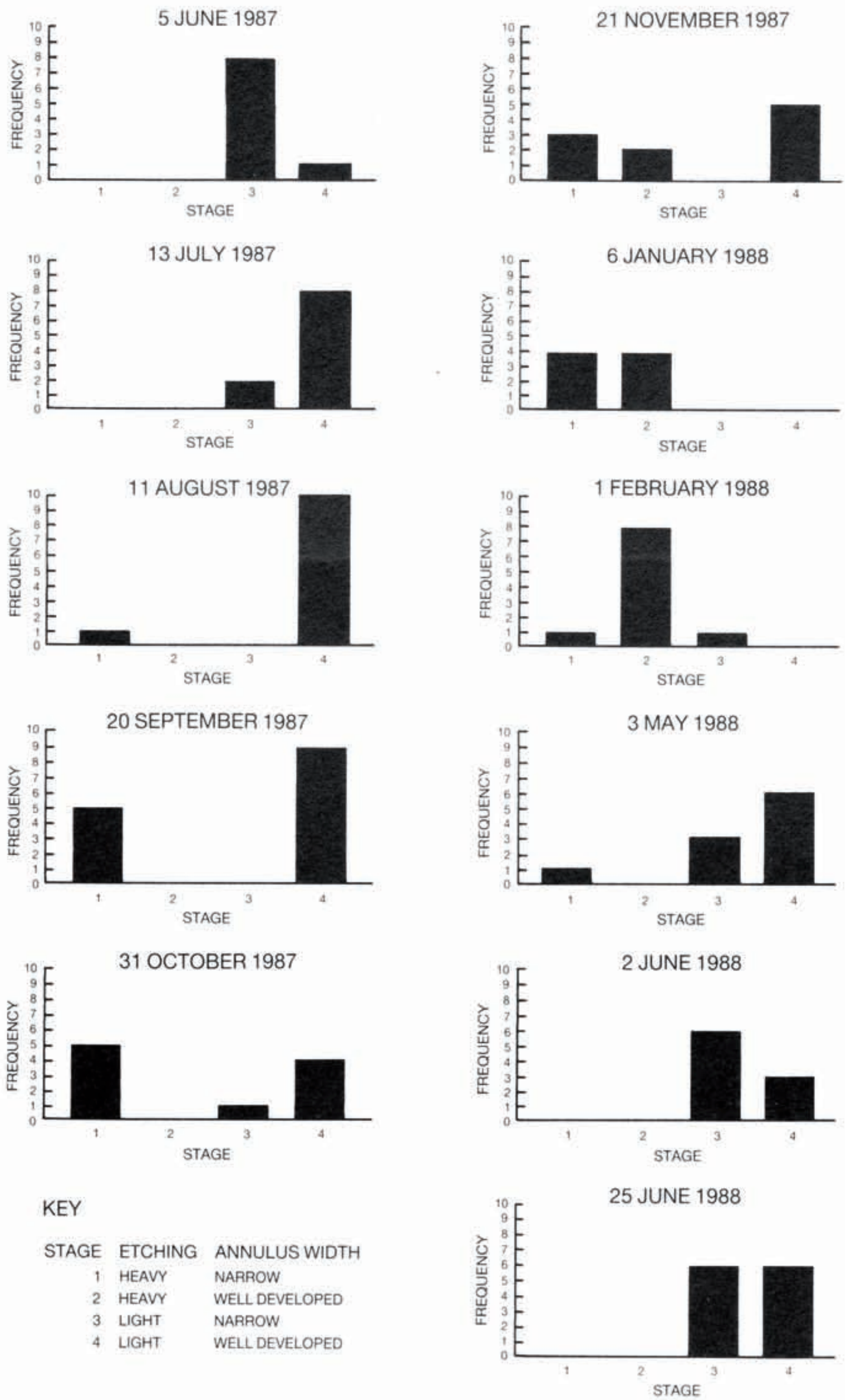


Fig. 4. - Histograms for growth stage of outer annulus revealed by SEM for monthly time series of samples of otoliths from *Notothenia coriiceps* showing progression of stages from June 1987 to June 1988.

In turn, super-imposing the images revealed by light microscopy and SEM techniques there was an obvious general coincidence in the stage of annuli development (Fig. 5), demonstrating that the former also represent one year. The annuli revealed by light microscopy consist almost entirely of an opaque zone and a very narrow hyaline zone.

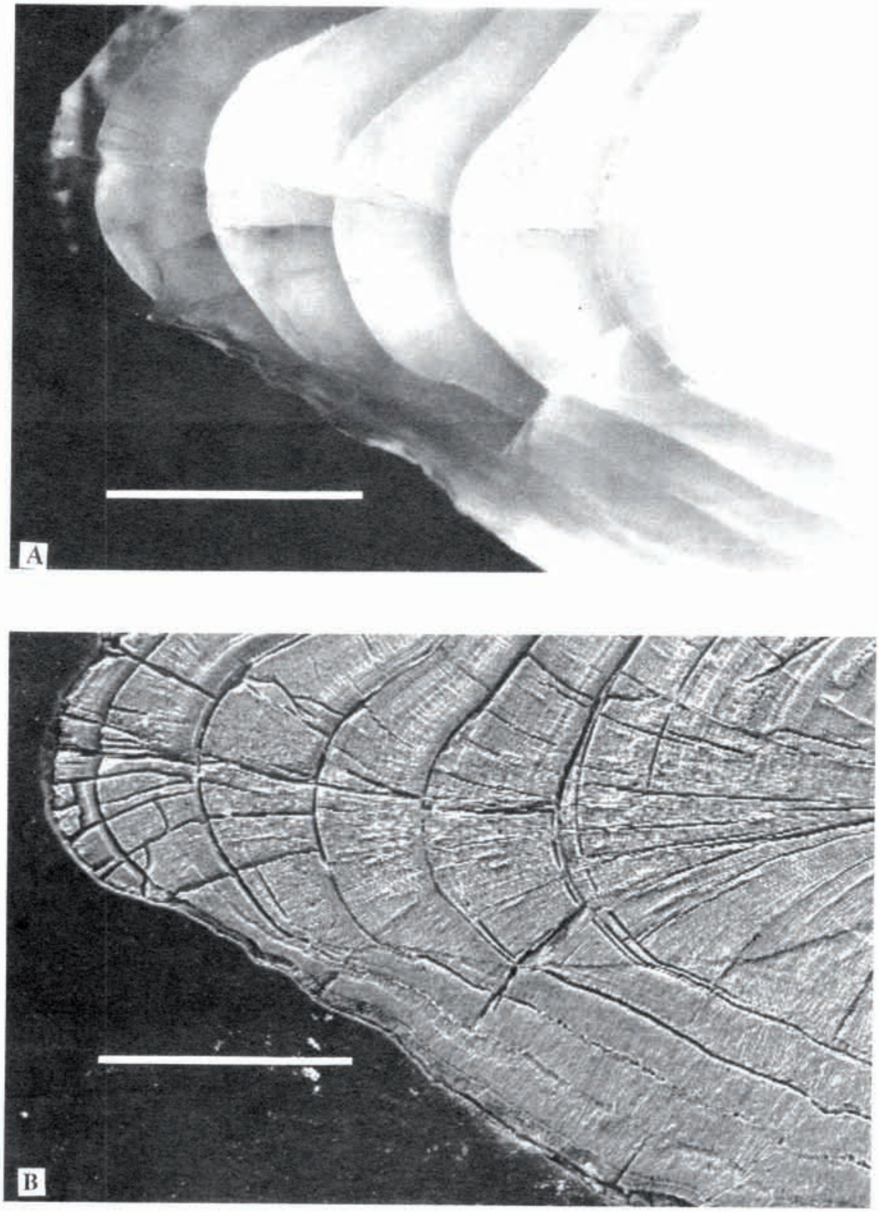


Fig. 5. - Structures observed for otolith from *Notothenia coriiceps* using light microscopy (A) and scanning electron microscopy (B), illustrating the general coincidence of annuli revealed by both techniques.

Based on measurements taken from defined marks visible in both images, we interpret the hyaline zone to correspond with either the end of SEM stage 4 or the beginning of SEM stage 1; the remaining growth, with stages 2 and 3, corresponds to the opaque zone. An exact correspondence between the hyaline zone revealed using light microscopy and the heavily-etched 'check' in the SEM image was not achieved.

DISCUSSION

Otolith structure in cross-section

In otoliths of Antarctic fish, differentiation of cross-sectional radial structure into regions has not been thoroughly investigated. In this study there was little evidence for the regions being derived from different primordia in the central nuclear core revealed by SEM, although the area of micro-increments immediately outside the nuclear core showed a characteristically asymmetrical compressed pattern. The structure and width of annuli were different in each region, but all showed micro-increments. The differences mean that growth must vary between otolith regions: daily growth may be slower in regions where annuli are narrow, or the duration of growth may be shorter in these regions. Age determinations using the outer annulus may therefore be influenced by the region selected while micro-increments of each region need to be validated before they can be used reliably in age estimation. Another implication is that comparisons in age between specimens of *N. coriiceps* need to be based on corresponding regions of the otolith. Similar questions about the nature of growth arise over the progressive reduction in width of annuli revealed using light and SEM techniques with increasing distance from the nuclear core.

Validation of annual structure

In an earlier study of *N. coriiceps* at Signy Island using the 'crack and char' technique of Christensen (1964), Everson (1970) found that opaque zones were laid down in spring and early summer whereas hyaline zones were formed in early winter. Daniels (1983) immersed otoliths whole in glycerine and found that an opaque border appeared between July and September in otoliths of *Harpagifer antarcticus* caught at the Antarctic Peninsula. Burchett (1983a) sectioned otoliths using the Bedford (1983) method and indicated that a true hyaline zone was present between May and November on the edge of otoliths of *N. rossii* at South Georgia. North (1988) immersed otoliths whole in cedarwood oil and used light microscopy techniques to examine the edges of otoliths from an assemblage of fish species mostly from South Georgia: he found opaque zones present from January to June, whereas hyaline zones were found during July to January.

In this study, the distribution of growth stages revealed by SEM through the year in figure 4 indicates that the broad timing of annulus growth is similar to that described by North (1988). However, the lack of accurate correspondence between structures revealed by SEM and light microscopy in this study, the error due to the pseudo-hyaline edge in North's (1988) and the differences between the samples examined, mean that a more precise comparison is not possible. For a more reliable conclusion, direct comparisons of material collected from assemblages at South Georgia and the South Orkney Islands should be undertaken. Furthermore, both this study and that by North (1988) investigated fish from several year classes. In cod from the North Sea, Williams and Bedford (1974) found that opaque zones were laid down earlier in younger fish than in older fish. Given that annuli in *N. coriiceps* become more narrow further from the nuclear core, a similar situation may also occur. The variation found in this study may therefore be significantly reduced by examining specimens from a single year class.

Radtke and Hourigan (1990) concluded that the annuli revealed using the SEM in *L. nudifrons* did not correspond to yearly intervals. They suggested that this may be

because Antarctic marine habitats undergo smaller fluctuations in temperature which may result in a lack of distinct annual growth structures. The presence of yearly annuli in other species exposed to a limited range of temperatures suggests large variations between species, and indicates that otolith growth in Antarctic fish may be considerably more complicated than the hypotheses by either North (1988) or Radtke and Hourigan (1990) suggest. There is therefore a requirement for a hypothesis for annulus formation in Antarctic fish which can accommodate the observed phenomena.

The SEM technique used in this study overcomes many of the difficulties and reduces subjective interpretation inherent in examining otolith edges using light microscopy. This is partly due to higher resolution and the avoidance of light artefacts but also due to the effect of etching at the otolith periphery. A clear pattern to the edge of the outer annulus was visible in specimens viewed using the SEM, whereas this was not always possible when viewed using the light microscope due to the pseudo-hyaline edge.

Light microscope techniques are generally employed by fisheries biologists because conventional SEM techniques are comparatively slow for processing the large numbers of otoliths taken in fishery studies and more costly. However, with the adaptation by Ashford *et al.* (1993) of the Bedford (1983) method of otolith preparation, the use of SEM for samples collected during surveys will be of value for otoliths that are small or difficult to interpret. This study demonstrates that SEM techniques can greatly assist the understanding and precision in interpretation of annuli in otoliths of Antarctic fish. The SEM therefore provides a high-acuity image with enhanced information which can greatly improve interpretation of the image revealed using light microscopy depending on the degree of which the correspondence of structures between light and SEM images can be established.

CONCLUSIONS

1. Annuli revealed by SEM and light microscopy in *Notothenia coriiceps* are annual.

2. In *N. coriiceps*, SEM images showed that lightly-etched bands in annuli were deposited from February to July and heavily-etched bands was present from August to February.

3. Scanning electron microscopy gave greater resolution and additional information in comparison with light microscopy for viewing incremental structures in otolith sections.

4. Otolith cross-sections of *N. coriiceps* when viewed using SEM have a differential radial structure with varying growth patterns between regions. Age determinations may be influenced by the region selected.

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